ELSEVIER

Journal of Chromatography B, 769 (2002) 89–95

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of gentamicin in urine samples after inhalation by reversed-phase high-performance liquid chromatography using pre-column derivatisation with *o*-phthalaldehyde

Abdulsalam I. Al-Amoud, Brian J. Clark, Henry Chrystyn*

Postgraduate Studies in Pharmaceutical Technology, School of Pharmacy, University of Bradford, Bradford, West Yorkshire BD7 1DP, UK

Received 16 July 2001; received in revised form 3 December 2001; accepted 17 December 2001

Abstract

Gentamicin and netilmicin (internal standard) were extracted from urine using C_{18} solid-phase extraction cartridges (94.3% recovery) and then derivatised with *o*-phthalaldehyde and 3-mercaptopropionic acid. The derivative was stable for >6 h. The mobile phase, methanol–glacial acetic acid–water (800:20:180, v/v), contained 0.02 *M* sodium heptanesulfonic acid, pH 3.4, and was passed at 1.0 ml min^{-1} through a C_{18} column with fluorescence detection (excitation 340 nm, emission 418 nm). The four main components of gentamicin (C_1, C_{1a}, C_2, C_{2a}) and netilmicin, the internal standard, were separated. Using the C_{1a} gentamicin peak, linearity was demonstrated from 0.5 to 10 μg ml^{-1} and the limit of detection was 75 μg l^{-1}. Following 80-mg oral, 40-mg intravenous and 80-mg nebulised administration, the mean (SD) gentamicin urinary excretion was zero, 38.27 (0.96) and 1.93 (0.28) mg, respectively. Despite the relatively low lung deposition following inhalation of gentamicin the assay developed can be used to quantify the low urinary concentrations. Using this assay it should be possible to carry out urinary pharmacokinetic studies to identify the relative lung deposition of gentamicin following different methods of inhalation.

Keywords: Derivatization, LC; Gentamicin

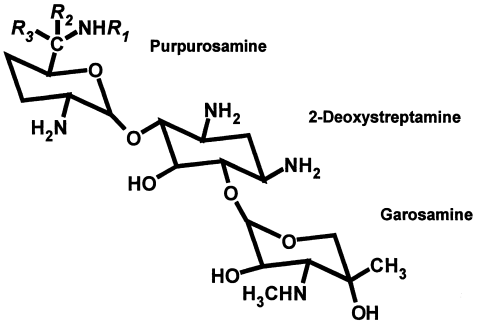
1. Introduction

Gentamicin is an aminoglycoside antibiotic produced from *Micromonospora purpura*. It is effective against a wide variety of a susceptible gram-positive and gram-negative bacteria [1], and is a mixture of four major components designated as C_1, C_2, C_{1a} and C_{2a}, which differ from each other in their degree of methylation on the purpurosamine ring [2]. The four major components, the structural formula, molecular masses and nomenclature of the amino sugar units comprising the gentamicin complex are shown in Fig. 1. Gentamicin is a highly polar polycationic molecule, which is relatively lipid-insoluble; therefore, it is very poorly absorbed from the gastrointestinal tract. The oral bioavailability is ~0.2% [3]. Gentamicin is eliminated by the renal route via glomerular filtration almost entirely in the active form [4]. In the management of systemic infections it is given by injection and for topical infections it is administered directly to the site. Hence for respiratory

*Corresponding author. Tel.: +44-1274-233-495; fax: +44-1274-236-490.

E-mail address: h.chrystyn@bradford.ac.uk (H. Chrystyn).

1570-0232/02/$ – see front matter
PII: S1570-0232(01)00636-5



Gentamicin Component	Peak no. in Fig 2b	R_1	R_2	R_3	MW
C_1	1	CH_3	H	CH_3	477
C_{1a}	2	H	H	H	449
C_{2a}	3	H	H	CH_3	463
C_2	4	H	CH_3	H	463

Fig. 1. Structural formula, molecular masses and nomenclature of the amino sugar units comprising the gentamicin complex.

infections delivery to the lungs by inhalation from a nebuliser is commonly used.

Numerous methods for the determination of gentamicin have been developed. These are based on a microbiological assay [5], enzyme immunoassay [6], polarization fluoroimmunoassay [7], gas–liquid chromatography (GC) [8], capillary electrophoresis [9] and high-performance liquid chromatography (HPLC). For the latter several modes of detection, including UV [10], fluorescence [11], electrochemical [12] and mass spectrophotometry [13], have been reported. The microbiological assay is inexpensive and simple, but the reproducibility is limited and it has a long incubation period. The immunoassay is more specific and accurate, but it depends on the purity of the enzyme. Both methods are not capable of quantifying the individual components of gentamicin and may be subject to possible interference from other antibiotics [14]. The HPLC methods with electrochemical detection and capillary electrophoresis methods are limited by their relatively high detection limits. Although mass spectrometry can allow very sensitive and specific measurement, it is unavailable to many facilities because of its relatively high cost.

Sensitive HPLC methods are not available because gentamicin does not possess an absorbing chromophore and, therefore, does not lend itself to UV or fluorescence detection. This necessitates derivatisation of gentamicin to allow its detection with the required sensitivity. Either pre or post-column derivatisation can implement this process. Previously gentamicin has been derivatised with *o*-phthalaldehyde (OPA) [15], dansyl chloride [16], fluorescamine [17], 9-fluorenylmethyl chloroformate (FMOC-Cl) [18] and 1-fluoro-2,4-dinitrobenzene (FDNB) [19]. Of these above methods the OPA derivatisation using tobramycin as the internal standard is the most promising. However this method has been developed for the assay of gentamicin in urine and serum samples from a rabbit. Furthermore the OPA derivative is unstable due to oxidation and although 2-mercaptoethanol is used to delay this, samples have to be assayed within 1 h. This method also uses derivatisation prior to extraction and thus reduces the batch size during analysis.

We have previously reported a method to determine the relative bioavailability of salbutamol to the lungs following inhalation by using urine pharmacokinetic studies [20]. A similar approach could

be possible for gentamicin after inhalation. The first stage of this new method is to develop a sensitive, simple, robust, efficient and reliable method for the quantitative estimation of gentamicin in urine samples collected after inhalation of the drug. 3-Mercaptopropionic acid is been used to prolong stability and netilmicin is used as the internal standard. The proposed method uses solid-phase extraction followed by derivatisation with OPA and 3-mercaptopropionic acid. Separation is achieved using C_{18} reversed-phase chromatography. This assay method was employed to identify the absolute lung bioavailability of gentamicin following nebulisation in human subjects.

2. Experimental

2.1. Materials

Gentamicin as Cidomycin® for injection, 80 mg in 2-ml ampoules, was obtained from Hoechst Marion Roussel (Uxbridge, UK). Heptanesulfonic acid (98%), *o*-phthalaldehyde (OPA) and 3-mercaptopropionic acid (3-MPA) were supplied by Sigma–Aldrich (Dorset, UK), netilmicin sulphate (Schering Plough, Herts, UK) was used for the internal standard. Glacial acetic acid, sodium hydroxide, ammonia solution and boric acid were purchased from BDH (Poole, UK) and methanol from Fisons (Loughborough, UK). All of these chemicals were of HPLC or analytical grade. Water was glass-distilled and further purified for HPLC using the Millipore Milli-Q system.

2.2. Gentamicin and netilmicin solid-phase extraction

A solid-phase extraction method using 6-ml cartridges packed with 600 mg octadecyl (C_{18}) (Spe™, J.T. Baker) to extract gentamicin and the internal standard (netilmicin) from urine samples was developed. The C_{18} cartridge was selected because it gave the most efficient sample clean-up with the highest recovery of gentamicin and netilmicin from urine samples. These cartridges were wetted with 2 ml methanol and conditioned with 2 ml distilled water. A 1 ml aliquot of human urine containing gentamicin and 0.5 ml of netilmicin sulphate (1.5 mg l^{-1}; internal standard) was then passed through the cartridges for 2 min, followed by a full vacuum for 5 min to dry the cartridges. The C_{18} cartridges were then washed with 4 ml distilled water and dried using a full vacuum for 3 min. Then 4 ml of methanol–water (50:50, v/v) was applied through the cartridges and again dried using a full vacuum for 3 min. Before the elution step, 4 ml of methanol was passed through each cartridge column to rinse the sorbent bed. The sorbent bed was then dried using a full vacuum for 3 min. Finally, the gentamicin and netilmicin compounds were eluted using 1 ml of a 10% (v/v) ammonia solution in methanol. The solvents were then evaporated using a stream of nitrogen.

2.3. Derivatisation procedure

Boric acid (24.736 g) was dissolved in 800 ml of distilled water, the pH was then adjusted to 10.4 using a 40% (w/v) solution of sodium hydroxide and sufficient water was added to produce 1000 ml. A stock solution of derivatising agent containing 256 mg *o*-phthalaldehyde dissolved in 50 ml methanol (methanolic–OPA solution) was prepared, 5 ml of this solution was mixed with 20 ml of the boric acid solution (pH 10.4) and mixed with 50 μl of 3-mercaptopropionic acid (3-MPA). This derivatising agent was stable for 5 days when stored at 4 °C in amber vials.

Following solid-phase extraction, the residue (after evaporation) was reconstituted by adding 0.25 ml of the derivatising agent and 0.75 ml of distilled water. This was vortexed for 30 s and 50 μl was injected into the HPLC system.

2.4. Equipment and analytical method

The HPLC system used was the Milton Roy CM 4000 multiple solvent delivery system (LDC Analytical, Stone, UK) linked to a Waters 470 (Millipore, USA) scanning fluorescence detector set at an excitation of 340 nm and an emission of 418 nm. The detector signal was relayed to a Hewlett-Packard HP 3394A integrator (Hewlett-Packard, Waldbronn, Germany). Aliquots (50 μl) were injected using a Promis II automatic sampler injection system fitted

with a 50 μl volume loop (Rheodyne-Waters, Milford, USA). Chromatographic separation was performed using Allsphere ODS-2 packing in a 25 cm×4.6 mm I.D (internal diameter) stainless steel column (Kinesis, Epping, UK) protected by a 3 cm×4.6 mm I.D. guard column packed with spherisorb ODS (Hichrom, Reading, UK).

The mobile phase used was methanol–glacial acetic acid–water (800:20:180, v/v/v) containing 0.02 *M* sodium heptanesulfonic acid as previously reported by Cabanes et al. [15]. The mobile phase was adjusted to pH 3.4 using acetic acid. The mobile phase was filtered under vacuum through a 0.45 μm filter (Gelman Sciences, Germany) and degassed in an ultrasonic bath under vacuum for 20 min. Gentamicin OPA derivatives were injected into the system and separated at ambient temperature using a constant flow rate of 1.0 ml min^{-1}. The four main components of gentamicin (C_1, C_{1a}, C_2 and C_{2a}) were assigned to the resolved peaks according to Cabanes et al. [15].

2.5. Inter- and intra-day variation

Pooled urine from five volunteers (three females and two males) was used to prepare all standard gentamicin solutions. For each of the urine samples the recovery was compared against aqueous standards and the determinations were carried out five times. Inter-day variability was performed for each standard concentration repeating the assay five times. Intra-day variability for each concentration was repeated on 5 consecutive days.

2.6. Volunteer study

A total of eight (four female) healthy volunteers gave written informed consent to take part in the study and local ethics approval was obtained. Their mean (SD) age and weight were 32.0 (8.0) years and 67.5 (15.1) kg, respectively. On separate study days (7 days apart), according to a randomised schedule, they each either swallowed 80 mg gentamicin in 20 ml of water, inhaled 80 mg gentamicin (made up to 4.5 ml with normal saline) from a Pari LC^+ nebuliser chamber driven by a PariBoy nebuliser (Pari, Germany) or received 40 mg of gentamicin given by intravenous bolus injection. Urine samples were collected at 0, 0.5, 1.0, 2.0, 4.0, 6.0, 9.0, 12.0, 24.0 and 36.0 h post dose.

3. Results

Fig. 2A shows typical chromatograms of a blank urine sample from a volunteer prior to nebulisation; the chromatogram is typical of that for each volunteer. Fig. 2B shows the four components of gentamicin, C_{1a}, $C_{1,}$ $C_{2,}$ C_{2a}, and netilmicin eluted as distinct peaks with retention times of 5, 11, 15, 16, and 18 min, respectively, from a standard prepared from the volunteers' urine. This shows that there are no interfering peaks when compared to the chromatogram of the blank urine samples. The gentamicin C_{1a} peak is well resolved and gives a sharp peak with a retention time of 11 min whilst that of netilimicin was 18 min. For this reason all the calculations for the quantification of gentamicin are based on the peak height of C_{1a} with respect to the internal standard. The C_{1a} component and the internal standard (netilmicin) were eluted from the column with capacity factors (k^1) of 4.45 and 9.10, respectively. Fig. 2C shows a chromatogram of one of the samples from the in vivo study. The gentamicin concentration in this sample is 5 mg l^{-1}.

The standard calibration curve for gentamicin in urine (range 0.5–10 mg l^{-1}) was linear, described by the equation $y=0.4986x+0.2465$ ($r=0.998$, $n=35$). The lower limit of detection (LOD) and the limit of quantitation (LOQ) were calculated from the mean (SD) of the intercept of four calibration curves. A value of greater than 3:1 for the signal-to-noise ratio was used for the LOD and of greater than 10:1 for the LOQ. The LOD and LOQ were 75 and 250 μg l^{-1}, respectively. The intra- and inter-day variation of the assay, determined by adding the appropriate amounts of gentamicin and netilmicin to pooled urine from five volunteers, is shown in Table 1. The recovery of gentamicin from urine when compared to corresponding aqueous standards was consistent and the mean (SD) absolute percentage recovery was 94.3 (1.72)% ($n=5$ for each of the seven gentamicin standards between 0.5 and 10 mg l^{-1}). OPA solution was used for five consecutive days and a new calibration curve was constructed every week using freshly prepared OPA solution.

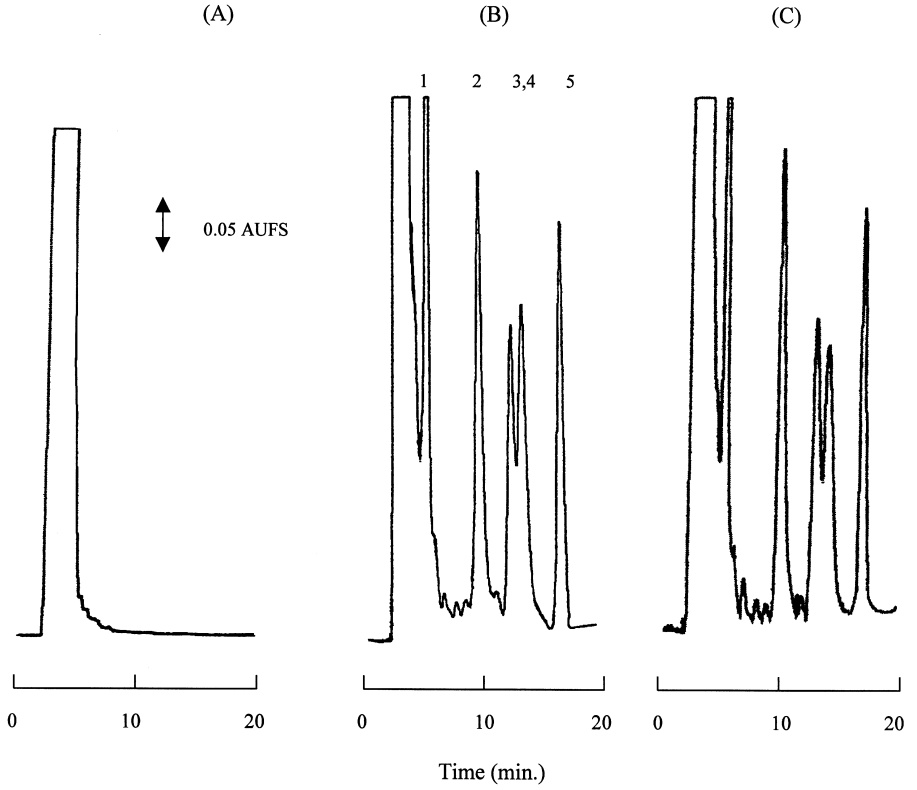


Fig. 2. Chromatogram of (A) blank urine sample, (B) standard urine sample containing 5 mg/l gentamicin and 1.5 mg/l netilmicin (1, gentamicin C_1; 2, $C_{1a,}$; 3, $C_{2,}$; 4, C_{2a}; and 5, netilmicin), and (C) urine sample of volunteer receiving 80 mg nebulised gentamicin.

No gentamicin was detected in any of the urine samples provided post oral administration and thus none was absorbed. Furthermore, no gentamicin was detected in any of the urine samples between 24 and 36 h after the nebulised dose. Fig. 3A,B describes the urinary excretion of gentamicin following intravenous and nebulised doses, respectively. The mean (SD) amount of gentamicin recovered in the urine following intravenous (40 mg) and nebulised administration (80 mg) was 38.27 (0.96) and 1.93 (0.28) mg, respectively. The mean (SD) ratio, representing the absolute bioavailability of gentamicin to the lung following the nebulised method, was 5.04 (0.73)%. Comparison of the C_{1a} peak to the other

Table 1
Precision of the assay for gentamicin in urine (intra- and inter-day variation) ($n=5$ for each determination)

Nominal concentration (mg/l)	Intra-day			Inter-day		
	Mean	SD	C.V. (%)	Mean	SD	C.V. (%)
0.5	0.45	0.02	5.44	0.45	0.03	6.32
1.0	0.95	0.04	4.63	0.95	0.05	5.15
2.0	1.94	0.06	3.15	1.89	0.09	4.83
4.0	3.82	0.13	3.36	3.78	0.16	4.25
6.0	5.63	0.16	2.84	5.74	0.26	4.54
8.0	7.57	0.24	3.22	7.53	0.41	5.41
10.0	9.67	0.34	3.56	9.48	0.46	4.84

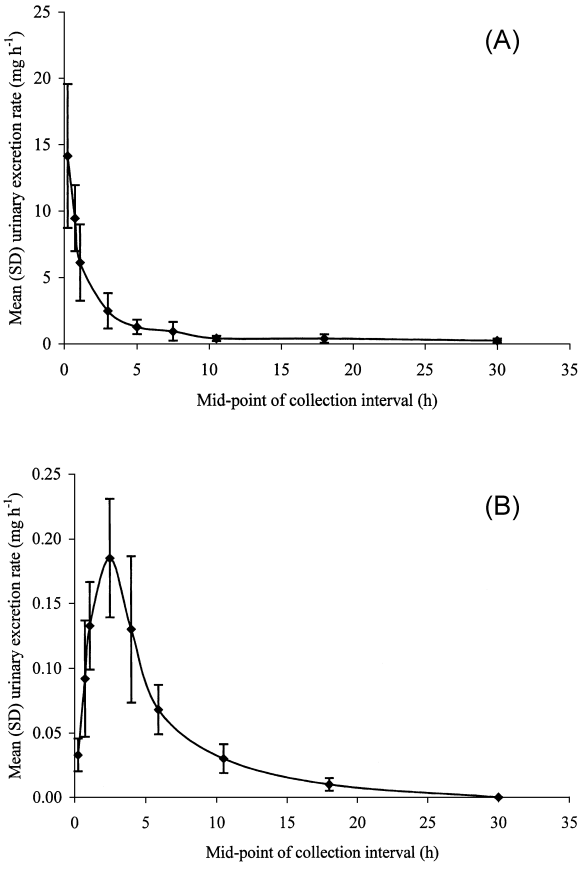


Fig. 3. Mean (SD) gentamicin urinary excretion rate post administration of (A) a 40-mg intravenous bolus and (B) 80 mg inhaled from a nebuliser ($n=8$).

gentamicin components highlighted a respective change for all the samples of each volunteer.

4. Discussion

Direct UV detection of gentamicin is impossible because of the lack of a UV chromophore and thus chemical derivatisation is necessary to enhance its UV absorbance. The derivatisation of gentamicin with dansyl chloride [16] requires heat and is time consuming. The procedure using FDNB [19] is also time consuming, requiring incubation of gentamicin with FDNB at 80 °C for 45 min. Derivatisation with fluorescamine [17] yields only a single peak for the gentamicin complex and derivatisation with FMOC-Cl [18] may be problematic since the derivatising agent is fluorescent (unlike OPA which is not fluorescent). OPA was chosen as the derivatising agent, because in an aqueous media the reaction occurs rapidly at room temperature. The use of 3-mercaptopropionic acid (3-MPA) with OPA provides a derivative with less fluorescence than the derivative with 2-mercaptoethanol reported by Cabanes et al. [15]. However 3-mercaptopropionic acid prolongs the stability of the derivative against oxidation. The stability lasts more than 6 h compared to 1 h for the previous method [15] and allows more samples to be analysed. The method has a slightly lower limit of quantification than that proposed by Cabanes et al. [15] but the difference is insignificant.

The derivative formed with tobramycin eluted early, together with some polar impurities from the extracted human samples. Netilmicin is a dehydrogenated analogue of gentamicin C_{1a} [21] and due to its close chemical similarity with other gentamicin components it serves as a useful internal standard. Solid phase extraction using a C_{18} cartridge has several advantages over liquid–liquid extraction, in that it provides cleaner extracts and removes any material that would otherwise adsorb irreversibly to the column matrix. Derivatisation is carried out post extraction which allows more time within which to quantify the gentamicin in an automated system. Furthermore, sensitivity can also be improved by increasing the sample size.

The intravenous data highlight a renal route for excretion of the drug delivered to the body and the respective changes in peak size of the gentamicin components to the C_{1a} peak used reveals that all components are eliminated at the same rate. Following inhalation drug is either deposited into the lungs or is swallowed. Since no gentamicin was detected in any of the urine samples post oral administration then the amounts appearing in the urine post inhalation would have been delivered to the body by the pulmonary route. Although an oral bioavailability of 0.2% has previously been reported [3], the dose used was much larger than that used therapeutically. Comparison between the amount of gentamicin excreted after the intravenous and inhaled doses reveals that the nebulised method delivered a mean (SD) of 5.04 (0.73)% (of the inhaled dose) to the lungs. This highlights that the nebulised method is an

inefficient system to deposit gentamicin into the lungs. Despite this low absolute lung bioavailability the assay developed has sufficient accuracy and robustness to enable pharmacokinetic studies to be used to identify amounts delivered to the lungs following gentamicin inhalations.

5. Conclusion

A rapid and sensitive method was developed for the determination of gentamicin in human urine after inhalation during pharmacokinetic studies. This HPLC method used post-column derivatisation of gentamicin with *o*-phthalaldehyde with the addition of 3-mercaptopropionic acid to increase the stability. The method is able to measure low concentrations of gentamicin. It is, therefore, well suited for performing pharmacokinetic studies from routes with low systemic bioavailability as highlighted by the nebulised method. The assay has an acceptable limit of both accuracy and precision and is capable of detecting gentamicin in urine at concentrations as low as 75 $\mu g\ l^{-1}$.

References

[1] M.J. Weinstin, G.H. Wagman, E.M. Oden, J.A. Marquez, J. Bacteriol. 94 (1967) 789.
[2] D.J. Cooper, M.D. Yudis, H.M. Marigliano, R.D. Cuthric, S.T.K. Bukhari, J. Chem. Soc. C (1971) 3126.
[3] A. Kucers, S.M. Crowe, M.L. Grayson, J.F. Hoy, in: The Use of Antibiotics: Gentamicin, Butterworth-Heinemann, Oxford, 1997, p. 450.
[4] J. Black, B. Calesnick, D. Williams, M.J. Weinstein, Antimicrob. Agents Chemother. 1 (1964) 138.
[5] L.D. Sabath, J.I. Casey, P.A. Ruch, L.L. Stumpf, M. Finland, J. Lab. Clin. Med. 78 (1971) 457.
[6] J.M. Andrews, R. Wise, J. Antimicrob. Chemother. 14 (1984) 509.
[7] S.A. Brown, D.R. Newkirk, R.P. Hunter, G.G. Smith, K. Sugimoto, J. Assoc. Off. Anal. Chem. 73 (1990) 479.
[8] J.W. Mayhew, S.L. Gorbach, J. Chromatogr. 151 (1978) 133.
[9] C.L. Flurer, K.A. Wolnik, J. Chromatogr. 663 (1994) 259.
[10] P.M. Kabra, P.K. Bhatnagar, M.A. Nelson, J. Anal. Toxicol. 7 (1983) 283.
[11] P.J. Kijak, J. Jackson, B. Shaikh, J. Chromatogr. 691 (1997) 377.
[12] L.A. Kaine, K.A. Wolnik, J. Chromatogr. 674 (1994) 255.
[13] L.G. McLaughlin, J.D. Henion, P.J. Kijak, Biol. Mass. Spectrom. 23 (1994) 417.
[14] B.H. Minshew, R.K. Holmes, C.R. Baxter, Antimicrob. Agents Chemother. 7 (1975) 107.
[15] A. Cabanes, Y. Cajal, I. Haro, J.M. Garcia Anton, M. Arboix, F. Reig, J. Chromatogr. 14 (1991) 1989.
[16] G.W. Peng, M.A.F. Gadalla, A. Peng, V. Smith, W.L. Chiou, Clin. Chem. 23 (1977) 1838.
[17] S.E. Walker, P.E. Coates, J. Chromatogr. 223 (1981) 131.
[18] D.A. Stead, R.M.E. Richards, J. Chromatogr. B 675 (1996) 295.
[19] W. Hospes, R.J. Boskma, J.R.B.J. Brouwers, Pharm. Week. Sci. Ed. 4 (1982) 32.
[20] M. Hindle, H. Chrystyn, Br. J. Clin. Pharmacol. 34 (1992) 311.
[21] J. D'Souza, R.I. Ogilvie, J. Chromatogr. 232 (1982) 212.